Quantitation of Papova Virus Particles in Human Warts [1, 2]

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SUMMARY

The quantitative determination of virus particles in human warts is described. Human papilloma (wart) virus belongs to the papova virus group whose members are easily recognized by their distinctive fine structures: spheres of 45 mμ diameter with deoxyribonucleic acid cores and a capsid exhibiting cubic symmetry. Among 40 warts tested, the highest virus concentration found was 7.3×10^9 particles per mg. The lower limit of counting sensitivity was about 10^7 particles per mg of wart tissue. The number of particles in the tissue was not associated with age or sex of the patient. There appeared to be a correlation, however, between the age of the wart and the concentration of particles, with warts 6 to 12 months old containing the highest concentration of particles. Large differences were found, however, in the concentrations of particles in warts of the same age.—J. Nat. Cancer Inst. 29: 583-595, 1962.

A NEED exists for quantitative work with tumor viruses, particularly for a method that will determine the total number of virus particles without dependence on bioassays. The problem is particularly acute in work on the viral etiology of human cancer because no such proved virus has yet been isolated in animal or tissue-culture systems. The human papilloma (wart) virus has been recognized morphologically since 1949 and was actually the first animal virus manipulated so as to form crystalline arrays (*1*, *2*). As this human virus is a member of the papova group of tumor viruses (*3*), we felt it important to apply recently developed particle-counting methods to its study in man, its natural host.

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Methods for particle counting were introduced by Sharp (*4*, *5*) and Backus and Williams (*6*). The method selected for this study was a modification of Sharp's agar sedimentation technique as recently described by Smith and Benyesh-Melnick (*7*).

MATERIALS AND METHODS

Wart samples were obtained from patients in the Houston area. After removal the warts were immediately placed in vials containing small volumes of tissue-culture medium with 100 units per ml of penicillin, 100 μg per ml of streptomycin, and 25 μg per ml of mycostatin. Vials were stored at −20° C for a few hours to 1 month before use.

Preparation of tissue homogenates was done with the micro-tissue extractor and shaking machine described by Sharp (*5*). Whole warts or pieces (in this instance having all the wart layers) weighing 5 to 30 mg were washed in sterile saline and drained well on a piece of sterile gauze for a few seconds. Then the tumor was cut with small scissors into pieces of about 1 mm^3. These pieces were put into the extractor (*5*) containing a drop of saline, the wet extractor having been weighed on the microbalance, and the total weight determined. By subtraction, the wet weight of the wart tissue was arrived at. Then a steel ball and 0.2 ml of sterile saline were put into the extractor that was closed with an aluminum plug. The extractor was placed in the shaking machine (*5*) and agitated at 60 strokes per second for 8 periods of 55 seconds each. We obtained a foamy paste that was quantitatively transferred to a sterile plastic tube by washing with several small samples of sterile saline. Each time saline was added the extractor was closed and shaken briefly. The total amount of saline varied with different samples, but the final concentration was usually 2.5 to 5.0 mg of wart tissue per ml of suspension.

The wart suspension was then sonically vibrated in a Raytheon sonic oscillator, 10 kc, model DF 101. Lusteroid cellulose tubes (0.5 × 2 in.), containing 1.5 to 3 ml of tissue homogenate, were rubber-stoppered and placed in the cup of the oscillator that was partially filled with water at room temperature. The wart suspensions were sonicated at maximum power for 60 seconds. Smooth, opalescent suspensions were obtained when sonic treatment followed immediately the preparation of the homogenate. Sometimes, however, the wart suspensions were left overnight in the freezer before sonication. Then sonic treatment was effective only if homogenates were previously thawed in the water bath at 37° C for 10 minutes.

Enzymatic treatment was the next step. The sonic-treated suspension, 0.2 ml, was mixed with 0.1 ml of 0.1 percent trypsin-chymotrypsin and incubated at 37° C for 20 minutes. The mixture was diluted 1 : 25 to 1 : 50 with sterile saline and was then ready to be sedimented onto the agar disc.

The remaining steps and the procedure for calculation of the concentration of particles per ml have been described previously (*7–9*). Usually, 5 micrographs were randomly taken for each count and the average number of virus particles present per unit area was used in the calculation. From the weight of the wart tissue in the suspension, a simple calculation gave the number of virus particles per mg of wart. The standardized counting method described had a limit of sensitivity approximating 1×10^7 virus particles per mg of wart tissue (an average of 1 particle on each of 5 micrographs).

Initial attempts with the micro-tissue extractor showed that the time of agitation necessary to obtain a homogenous suspension may vary from one wart to the next. Eight periods of agitation of 55 seconds each were used on all warts in this study, as this was found to homogenize even the most resistant.

We used enzyme treatment to avoid the masking of virus particles by tissue debris. Perhaps this could have been avoided by differential centrifugation or by dilution of the wart sample. However, as discussed later, centrifugation often removes a large fraction of virus particles due to aggregation, and dilution is not feasible for samples with low virus counts. Suspensions digested with trypsin-chymotrypsin for 30, 45, and 60 minutes gave approximately equal counts of virus particles. Thus, particles were not degraded by enzyme treatment even 3 times longer than the 20-minute period adopted for routine use.

Two experiments were done to study the effect of sonic vibration on virus-particle counts. Samples were sonic treated for 30 and 120 seconds, enzyme treated, counted, and the results compared with non-sonicated controls. Slightly higher counts were obtained from sonicated specimens, which indicated some further release of particles from tissue debris. This was a safe procedure, for sonic treatment up to 120 seconds could be used without causing a detectable loss of countable virus particles. All warts in this study were sonicated for 60 seconds and then digested for 20 minutes at 37° C.

RESULTS

A chromium-shadowed preparation containing large numbers of the type of particle counted in this study is shown in figure 1. Most of the particles are flattened, casting short shadows. Some particles have depressed centers, giving the appearance of a mammalian erythrocyte. Isolated particles display this flattened appearance more distinctly. These features were rather constant throughout most of the preparations examined and were especially useful for identification when particle numbers were small. When specimens with large numbers of particles were found, they almost invariably contained many large aggregates. These aggregates commonly contained 10 to 100 particles and were almost always

in a single plane. This arrangement of aggregated virus made counting much simpler than if the arrangement had been in several planes.

Selected preparations containing large numbers of particles were stained with uranyl acetate. Staining was done by the method described by Smith and Melnick (*9*) using crude samples prepared in the routine way. Most particles stained intensely in the core area (fig. 2). The hexagonal outlines of some particles are clearly seen and those staining in the core area are easily identifiable. However, particles that do not display this core stain are rather difficult to see (fig. 2). The same wart extract described previously was stained with potassium phosphotungstate (*p*H 5.8). Figure 3 shows the particles prepared in this way. The capsomeres are clearly seen, and the arrangement and number are similar to that described by Williams *et al.* (*10*). Most particles were not penetrated by the phosphotungstate, and reflected the presence of cores in the majority of them.

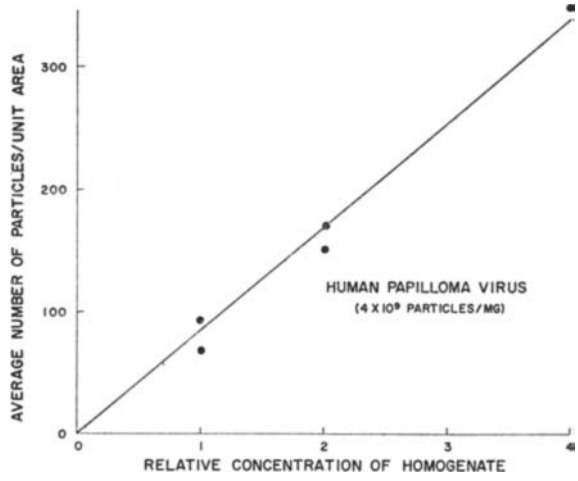
To determine whether particle counts of serially diluted preparations were proportional to relative concentrations, we performed the following experiment: A wart suspension containing 4×10^9 particles per mg was treated as described in Materials and Methods and diluted 1:25, 1:50, and 1:100 with saline. One plate with micrographs of 5 fields *chosen at random* was taken of the preparation diluted 1:25. Two plates (micrographs of 10 fields also chosen at random) were taken of each preparation that had been diluted 1:50 and 1:100. The results are shown in table 1 and text-figure 1. Similar results were obtained with two other suspensions tested in the same way. It is concluded, therefore, that the standardized counting procedure is valid and reasonably precise.

The counting procedure described previously was used for the determination of the concentration of virus particles in 40 common warts, 30 from males and 10 from females. The ages of the patients ranged from 9 to 74 years, with 30 of them from 15 to 36 years. Thirty specimens were taken from the hand, 5 from the foot, and 5 from other locations (groin, knee, and elbow). The ages of the tumors varied from 2 weeks to 9 years. The results, presented in table 2, are arranged from the highest to the

TABLE 1.—Particle counts from individual electron photomicrographs of a serially diluted human papilloma-virus preparation

	Dilutions				
	1:25	1:50		1:100	
Micrograph No.	(One plate only)	Plate 1	Plate 2	Plate 1	Plate 2
1	303	181	62	53	60
2	410	149	97	90	50
3	406	127	3*	174	73
4	422	167	416	44	41
5	196	131	102	104	115
Average per micrograph	347	151	169	93	68

*Obviously a mistake occurred here, and this count was disregarded in the calculation of the average.



TEXT-FIGURE 1.—Relationship between particle counts and relative concentration of human wart virus.

lowest concentration of virus particles found in individual specimens. The sex and age of the patient, location of the wart, age of the wart, and particle count per mg of tumor are given. The smallest amount of virus counted was 1×10^7 particles per mg (lower limit of sensitivity) and the greatest was 7.3×10^9 particles per mg. Thus, the range of concentrations

TABLE 2.—Virus particles in human warts

Sample No.	Patient		Wart		Particles per mg of wart ($\times 10^7$)
	Sex	Age	Location	Age	
1	M	41	Finger	6 Months	730
2	F	48	Finger (palmar)	6 Months	400
3	M	19	Palm	11 Months	76
4	M	23	Finger (palmar)	6 Months	64
5	M	74	Unknown (filiform)	Few weeks	56
6	M	22	Finger	1½ Years	48
7	M	9	Plantar	2–3 Months	32
8	F	19	Finger	2–3 Months	30
9	M	24	Finger	8–9 Months	30
10	M	30	Knee	2 Years	30
11	M	18	Toe	1 Month	20
12	M	18	Foot (side)	2 Months	20
13	M	35	Groin	Few weeks	15
14	F	26	Finger	2 Years	10
15	M	18	Palm	2 Years	10
16	M	18	Palm	2 Years	7
17	F	48	Finger	1 Month	6
18	M	42	Finger (dorsal)	3 Years	6
19	M	20	Finger (dorsal)	2 Years	5
20	F	15	Finger	1 Year	4
21	M	42	Finger (dorsal)	3 Years	2
22	M	18	Finger (dorsal)	4 Years	1
23 to 40	F (5)	12–49	Fingers	2 Weeks–1 year	Negative
	M (13)	18–31	Fingers, plantar, elbow	2 Months–9 years	Negative

observed was 730-fold. Of the 40 warts, 22 (55%) contained 1×10^7 or more particles per mg, 18 (45%) containing less than this lower limit for counting.

Positive samples were found in warts from a few weeks to 4 years old, from any location, and from patients of both sexes ranging in age from 9 to 74 years. Negative specimens (those having no detectable particles with the method used) were found in warts 2 weeks to 9 years old, from any location, and from patients of both sexes ranging in age from 12 to 49 years.

The effect of long storage at −20° C of warts known to contain virus particles was determined. Extracts were prepared from pieces of a single wart and counted 5 months apart. They were found to contain 3.6×10^8 and 2.8×10^8 particles per mg, respectively. It appears, therefore, that warts may be stored for several months at −20° C without large losses of countable virus particles.

DISCUSSION

Three things were considered in this study: particle recognition, particle quantitation, and the application of a standardized counting method to determine the concentration of virus particles in individual tumors. Human wart virus particles are of "medium" size in relation to other viruses and have a uniform morphology. The cubic symmetry displayed by each particle is a further advantage in its identification. Shadowed particles are usually flattened, and isolated particles are reasonably easy to identify by their slightly angular outline and erythrocyte-like appearance. Uranyl acetate and phosphotungstate staining (*9*) can be used for further establishment of the identity of suspicious particles as viruses. The older problem of what to count in the electron microscope has therefore become much less troublesome.

Clarifying low-speed centrifugation of tissue extracts was carefully avoided in this study because the measured diameters of many particle aggregates were as high as 5 to 10 μ, or the size of red blood cells. Centrifugation of such preparations for 10 minutes at $200 \times g$ would probably remove most such aggregates, which would cause a tremendous intrinsic error in quantitation. The extracts resulting from agitation in the Sharp Lucite chamber (*5*) and brief sonic oscillation were macroscopically homogeneous. Enzymes further reduced most of the already small tissue debris to sizes not readily spun down during the centrifugation required for sedimentation of the virus particles and their subsequent counting in the electron microscope.

The limit of sensitivity of the counting method was imposed by the necessity of dilution of the enzyme-treated crude extracts sufficiently to minimize the effects of remaining debris. Further purified suspensions could be counted with a somewhat higher sensitivity, but most physical methods of purification involved some loss of virus particles during the

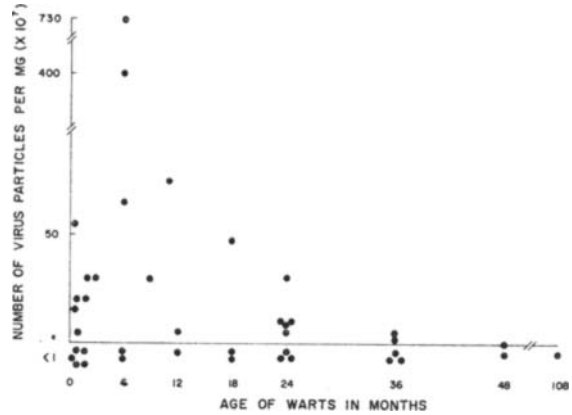
procedure. The relatively direct method described here was specifically designed to recover as nearly as possible all the virus particles present in the original tumor.

The data presented in table 2 show no correlation between the virus content of warts and the sex of the patient. Of the males, 17 of 30 (57%) and, of the females, 5 of 10 (50%) had warts yielding at least 10^7 particles per mg. These concentrations are not significantly different. Since 30 of the 40 warts examined were taken from the hand, no conclusions can be drawn from our study as to the relationship between location of the wart and the virus content. Similarly, most of the patients fell in the 15- to 36-year age group, which made the correlation between the age of the patient and particle content of the wart difficult. The 2 warts having the highest concentration of virus were taken from patients in their late forties, but the next 4 highest came from patients ranging in age from 19 to 74 years. There is, therefore, no obvious relationship between the age of the patient and particle concentration in the wart.

When one considers the relationship between the age of the individual wart and concentration of virus, negative and positive warts were found at practically all ages. Warts of similar age had great variations in their content of virus particles. Warts under 1 year of age tended to have more particles than older warts, and maximum individual counts were found in those warts ranging from 6 to 12 months of age. Text-figure 2 illustrates these points.

The earlier work of Strauss, Bunting, and Melnick (*11*) was based on qualitatively obtaining crystalline arrays of virus particles from human warts and not on quantitative estimates of virus particles, but their general conclusions on finding virus more readily in young warts agree with those drawn from the data presented here.

The counting procedure described quantitated particles over a range of 1 to 730 $\times$ 10^7 particles per mg of wart tissue. The technique apparently is sufficiently reliable as a routine procedure. It is a semimicro



TEXT-FIGURE 2.—Relationship between the age of warts and the concentration of virus particles per mg of wart tissue. Individual counts are shown as *dots*.

method and can be used to count virus in very small amounts of tissue. Thus one can screen large numbers of warts to find those containing high concentrations of particles without having to use significant amounts of material in the counting procedure. Purification of pools of such selected warts would be much easier because of the relatively small amount of contaminating tissue debris. As an illustration, warts #1 and 2, described in table 2, together contained about 71 percent of the total particles found in the 40 warts obtained for this entire study. If only these 2 warts were kept and the remainder rejected, a mere 29 percent loss of total particles would be traded for an initial source that would have been of 95 percent greater purity.

The number of Shope papilloma particles per infective dose, median (ID50) has been estimated at 10^8 (*12*). If there is a similar high ratio for the human papova virus, it would be important for one to select warts containing high virus concentrations for infectivity studies (inoculation of tissue cultures, infectious nucleic acid extraction, or passage *in vivo*).

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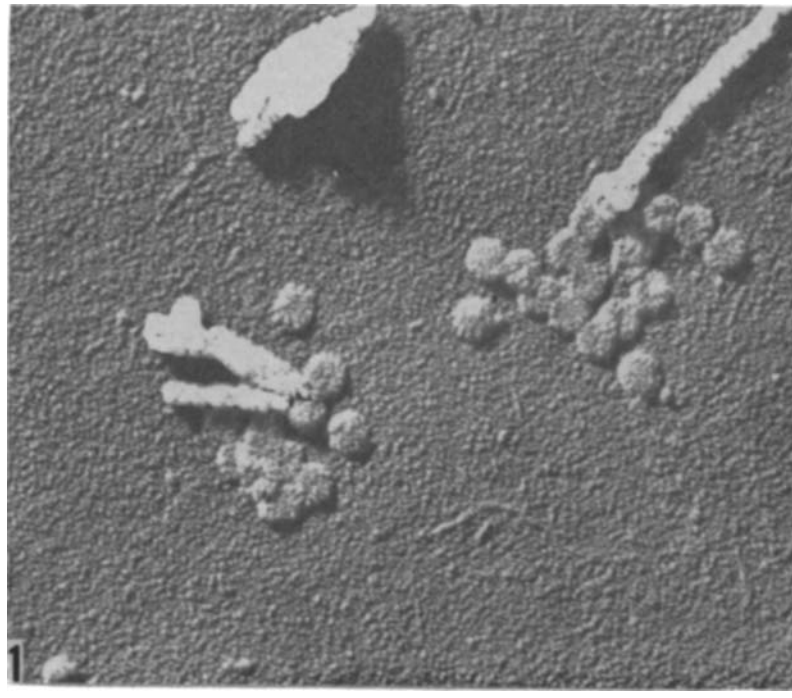
PLATES

PLATE 111

FIGURE 1.—Chromium-shadowed human wart virus prepared for particle counting. × 115,000

FIGURE 2.—Uranyl acetate-stained human wart virus. × 115,000



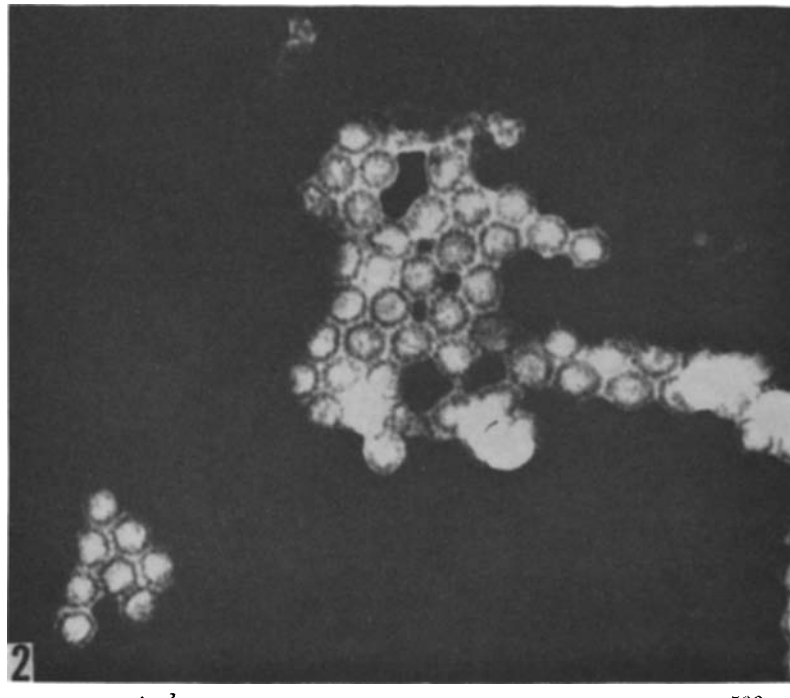
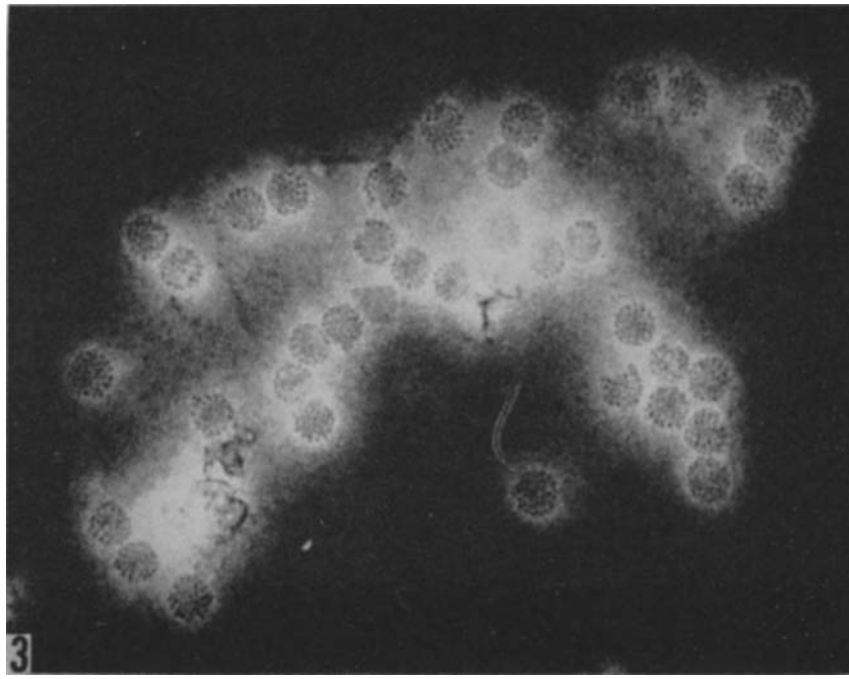


PLATE 112

FIGURE 3.—Phosphotungstate-stained human wart virus. × 115,000



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